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Evaluation of the Antimicrobial Activity of *Zanthoxylum zanthoxyloides* Root Bark Extracts*

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ABSTRACT

The development of resistance to antibiotics by infectious agents has been a continuous challenge. Thus, in this study, the aim was to evaluate the antimicrobial activities of *Zanthoxylum zanthoxyloides*, a potential plant source for novel antibiotics. Toward this end, dried powdered samples of the root barks of *Z. zantho*xyloides were extracted successively to obtain Crude Petroleum Ether (CPE), Defatted Ethanol Ether (DEE) and Defatted Ethanol Chloroform (DEC) extracts. The antimicrobial activities indicated by the size of the Zone of Inhibition (ZOI) of each extract at concentrations 5, 10, 15, 20 and 30 $\mu g\ \mu L^{-1}$ were evaluated against *Escherichia coli* (*E. coli*), methicillin-susceptible *Staphylococcus aureus* (MSSA), Methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus faecium* (VREF) using disc diffusion method. Two sets of Analysis of Variance (ANOVA) were performed. The first set comprised separate ANOVAs for each microorganism because the positive controls were different for each microorganism, although the negative control (DMSO) was the same for all. The second set was a single combined ANOVA with all microorganisms included with their positive controls excluded. The first set of analysis showed that DEE had significantly ($p<0.001$) higher antimicrobial activity than DMSO, CPE, or DEC. No significant interaction between extract and concentration was detected. The second set indicated a significant ($p<0.01$) interaction effect between extract and microorganism. Although no significant differences in ZOI were observed for microorganisms exposed to DMSO, CPE and DEC; one particular microorganism VREF was found to be the most susceptible to DEE. In addition, findings of this study show the potential of *Z. zanthoxyloides* as a source of broad-spectrum antimicrobial compounds.

Key words: Antimicrobial, *Zanthoxylum*, extracts, methicillin, vancomycin

INTRODUCTION

In addition to the alarming increase in the incidence of new and re-emerging infectious diseases, one major health concern is the resistance to existing antibiotics (Khanahmadi *et al.*, 2010; Agbafor *et al.*, 2011). Furthermore, novel antibiotics in the drug-development pipeline that offers significant benefits over existing drugs is lacking (Butler and Cooper, 2011). Examples of

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microorganisms that have developed antibiotic resistance were methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus faecium* (VREF). *S. aureus*, has acquired resistance to **β**-lactam antibiotics, such as penicillin and methicillin shortly after they have been used for treatment (Kobayashi and DeLeo, 2011). Due to its virulence, its development of resistance to antibiotics and its generally expensive drugs and treatment, MRSA has become a serious public concern worldwide (Akhi *et al*., 2008; Monecke *et al*., 2011). In the US enterococci rank second to staphylococci as the most common cause of nosocomial infections. In addition, among the *E. faecium* recovered from US hospitals, more than 80% are vancomycin-resistant (Panesso *et al*., 2010). At present the clinical development of potential drugs against gram-negative nosocomial infections that are significantly better than the existing drugs are still limited. The gram-negative bacteria are difficult to kill because they possess an additional outer membrane permeability barrier with multiple efflux pumps and antibiotic modifying enzymes (Butler and Cooper, 2011). As of this reporting, there is a sustained and urgent need to discover new antimicrobial compounds with novel action mechanisms.

It is widely known that plants possess healing properties (Cowan, 1999; Imaga, 2010; Agbafor *et al*., 2011). Such properties can be partly attributed to the diverse array of secondary metabolites (i.e., alkaloids, terpenes, phenolic compounds and cyanogenic glycosides) which are known to be essential for plants' defense against microbial attack, or insect and animal predation (Cowan, 1999; Dixon, 2001; Kido *et al*., 2010; Oseni and Akindahunsi, 2011). Treatment of common infections with medicinal plants has been popular in developing countries due to its cheaper cost and claims for both its effectiveness and lesser side effects over synthetic drugs (Rojas *et al*., 2006; Alagesaboopathi, 2010; Dey *et al*., 2010; Butkhup and Samappito, 2011; Karim *et al*., 2011; Menghani *et al*., 2011). A diverse range of compounds that offer potentials for the treatment of chronic and infectious diseases can be found most especially in traditional medicinal plants (Duraipandiyan *et al*., 2006; Karim *et al*., 2011; Sarwar *et al*., 2011). Well-known drugs that were derived from plants are taxol from *Taxus brevifolia*, vinblastine and vincristine from *Catharanthus roseus*, benzoin from *Styrax tonkinensis* and quinine from *Cinchona pubescens* (Mans *et al*., 2000). It is also well recognized that traditional medicine can be used alongside synthetic pharmaceutical products for enhanced health management (Imaga, 2010).

Zanthoxylum zanthoxyloides Lam also known as *Fagara zanthoxyloides*, is an indigenous plant used widely as chewing stick for tooth cleaning in West Africa (Adebiyi *et al*., 2009; Adegbolagun and Olukemi, 2010). Several studies on the various effects of its extracts have been reported. For example, Kassim *et al*. (2005) reported the anti-malarial activity attributed to benzophenanthridine alkaloid, fagaronine from *F. zanthoxyloides*' root extracts. Anti-malarial activity was also reported in a study using extracts from trunk barks of *F. zanthoxyloides* (Gansane *et al*., 2010). On the other hand, Patel *et al*. (2010) named the compound nitidine as the agent in *F. zanthoxyloides*' anticancer capabilities while an anti-inflammatory property due to ortho-hydroxymethyl benzoic acid made *F. zanthoxyloides* useful in the management of pain in sickle cell crisis (Oyedapo and Famurewa, 1995; Folasade *et al*., 2006). More recently, the potential of *Z. zanthoxyloides* leaf, bark and root extracts as a biopesticide for stored food protection has been reported (Udo, 2011). Due to the reported biological activities present in *Z. zanthoxyloides* the aims of the present study were (1) to evaluate the antimicrobial potential of *Z. zanthoxyloides* against bacterial pathogens that are of human health concern and (2) to determine if *Z. zanthoxyloides* can be a source of new antimicrobial compounds.

MATERIALS AND METHODS

This study was conducted from February 2010-April 2011. Sample collections and plant extractions were done at the School of Physical Sciences, Department of Chemistry, University of Cape Coast in Ghana, while the antimicrobial activity assays were done at the Department of Biology and Chemistry, Texas A and M International University.

Plant material: *Z. zanthoxyloides* root samples were collected from areas in the Amamoma forest, Cape Coast Metropolis in the central region of Ghana. The samples were examined and authenticated by Mr. Agyarkwah of the Herbarium Unit at the Botany Department of the University of Cape Coast. The root barks were thoroughly washed with water, peeled and chopped into small pieces. These were then sun-dried for ten days, milled into powder and kept in black polythene bags in a cool dry place for about 24 h.

Preparation of plant extracts: The dried powdered sample of the root barks was extracted successively (Fig. 1) to obtain (1) Crude Petroleum Ether (CPE), (2) Defatted Ethanol Ether (DEE) and (3) Defatted Ethanol Chloroform (DEC) extracts. The dried powdered sample of the root barks was defatted using petroleum ether (60-80°C) in a continuous soxhlet extractor until the solvent became colorless. A yellowish-brown extract obtained from extraction procedure was concentrated by steam drying using an evaporating dish resulting in a dark brownish paste. This was the Crude Petroleum Ether (CPE) extract.

The defatted sample was air-dried to remove traces of solvent and was exhaustively extracted with 95% ethanol. These ethanol extracts were evaporated to dryness, shaken with 2 M HCl and

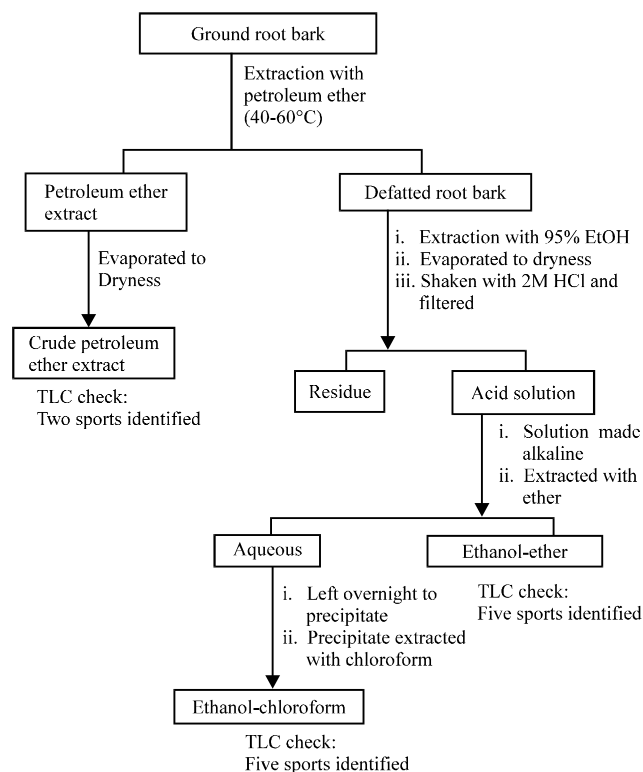

Fig. 1: Schematic diagram of crude extraction for *Z. zanthoxyloides*

then filtered. The filtrates were made alkaline by 28% NH_3 and then extracted by ether obtaining organic ether (Defatted Ethanol Ether (DEE) extract) and aqueous phases. On the other hand, the aqueous phase was left overnight to precipitate and then extracted by chloroform obtaining the Defatted Ethanol Chloroform (DEC) extract. The extracts, CPE, DEE and DEC were lyophilized using a Labconco freeze-dry system. Lyophilized samples were dissolved in dimethyl sulfoxide (Sigma) to obtain 5, 10, 15, 20 and 30 $\mu g\ \mu L^{-1}$ concentrations.

Microorganisms used: Antimicrobial studies were carried out using gram negative bacterium, *Escherichia coli* strain A 3379A Presque Isle Culture and gram positive bacteria, methicillin-susceptible *Staphylococcus aureus* 4651 Presque Isle Culture, methicillin-resistant *Staphylococcus aureus* ATCC 700699 and vancomycin-resistant *Enterococcus faecium* ATCC 700221.

Inoculum preparation: A single colony of each of the microorganisms was selected from the respective agar plate culture and transferred into a tube containing 2 mL nutrient broth. Using a water bath shaker, the broth culture was incubated at 37°C for 16 h. Using sterile nutrient broth, the turbidity of the actively growing broth culture was adjusted to a level of $A = 0.132 \pm 0.005$ at 625 nm. This level is optically comparable to the 0.5 McFarland standards. A spectrophotometer (Bausch and Lomb, Model Spectronic 20) was used to adjust the absorbance of the suspension. After having adjusted the turbidity of the suspension, a 1:1 dilution was prepared and used for inoculation into the Mueller-Hinton agar plates. This yields a bacterial suspension of approximately $0.5\text{-}1.0 \times 10^8$ CFU mL^{-1}.

Determination of antimicrobial activity: Antibacterial activity of plant sample extracts was determined via paper disc diffusion method (Bauer *et al*., 1966). As for the medium, a Mueller-Hinton agar was prepared from commercially available dehydrated base (Carolina Biological) according to manufacturer's instructions.

Moisture-free Mueller-Hinton agar plates were inoculated with 100 µL of the respective inoculum. The extracts impregnated in 6 mm disc filter paper discs (Flinn Scientific) were applied into the plates. Each disc had 20 µL of the plant extracts or DMSO. The DMSO used as the solvent in the preparation of different concentrations of the extracts served as the negative control to ensure that the solvent was not inhibiting the bacterial growth. On other hand, for (1) *E. coli*- streptomycin (10 µg) and kanamycin (30 µg) (2) *S. aureus*- penicillin (10 units) and novobiocin (30 µg) (3) methicillin-resistant *S. aureus*- penicillin (10 units but should be susceptible to penicillin) and novobiocin (30 µg) (4) *E. faecium*- penicillin (10 units) and chloramphenicol (30 µg) antibiotic discs (Flinn Scientific) were used as antibiotic positive controls (APC-1 and APC-2 for each microorganism, respectively). The plant extracts were used at 100-600 µg discs concentrations. The antibiotic positive controls (1) streptomycin and kanamycin in gram-negative bacteria i.e., *E. coli* and chloramphenicol in gram-positive bacteria i.e., *S. aureus* inhibit protein synthesis (Wisseman *et al*., 1954; Goldemberg and Algranati, 1981; Inoue *et al*., 2004), (2) penicillin inhibits cell wall synthesis in gram-positive bacteria i.e., *S. aureus* but not the methicillin-resistant *S. aureus* (Spratt, 1977) and (3) novobiocin inhibits DNA supercoiling in gram-positive bacteria *S. aureus* (MSSA and MRSA) (Gellert *et al*., 1976).

After incubation of the plates for 18-20 h at 37°C, all plates were observed for zone of inhibition. These zones were measured in millimeters using a Vernier caliper. All tests were carried out by quadruplicate and repeated three times.

Experimental design and statistical analysis: Two sets of statistical analysis were performed on the response variable, size of the zone of inhibition (ZOI in mm). The first set was an Analysis of Variance (ANOVA) associated with a 6×5 factorial experiment for each of the four microorganisms (i.e., *E. coli*, MSSA, MRSA and VREF). In this set, type of extract (six types, namely: DMSO, CPE, DEE, DEC, APC-1, APC-2) was one factor in which APC-1 and APC-2 were antibiotic positive controls for each of the microorganisms and level of concentration (five levels, namely: 5, 10, 15, 20 and 30) was the other factor.

Statistical analysis: The second set was an analysis for the combined data across microorganisms. The inclusion of microorganism as a factor resulted to a 4×4×5 ANOVA. The three factors were type of extract (DMSO, CPE, DEE and DEC), microorganism (*E. coli*, MSSA, MRSA, VREF) and concentration (5, 10, 15, 20 and 30) which translates to 80 treatment combinations in each complete block (Dowdy and Wearden, 1983; Quinn and Keough, 2002; Field, 2009). In this set, APCs (APC-1, APC-2) were excluded in the analysis because each microorganism had a different set of APCs. The actual time that each of the three replications was performed comprised the blocks (i.e., week 1, week 2 and week 3). All analyses were carried-out using the general linear model facility of the software Statistical Packages for the Social Sciences version 18.0 (SPSS, Chicago, Illinois). To identify which of the extract means were or were not significantly different, a Duncan's Multiple Range Test (DMRT) was employed (Field and Miles, 2010). To compare the 12 interaction means, 95% confidence intervals for each of the microorganism-extract combinations were calculated whereby overlapping intervals meant no significant difference.

RESULTS AND DISCUSSION

Inhibitory activity of extracts for each microorganism: In this study only the organic extracts, (1) Crude Petroleum Ether (CPE), (2) Defatted Ethanol Ether (DEE) and (3) Defatted Ethanol Chloroform (DEC) were investigated for their antimicrobial activities with DMSO and with APC-1 and APC-2 serving as negative and positive controls, respectively. This is due to the lack of significant antimicrobial activity by aqueous extracts from several plant species including *Z. zanthoxyloides* that have been reported from previous studies (Rotimi *et al*., 1988; Rojas *et al*., 2006; Doughari and Okafor, 2008; Ghadin *et al*., 2008; Zampini *et al*., 2009; Dey *et al*., 2010). The comparison of extract means was done by performing separate sets of ANOVA and DMRT for each of the microorganism.

Table 1 presents the mean comparisons of the organic extracts (CPE, DEE, DEC), the negative (DMSO) and the positive controls (APC-1, APC-2) with respect to the size of the Zone of Inhibitions (ZOI). Among the three organic extracts, DEE showed the greatest zone of inhibitions against the four microorganisms tested which were significantly higher at the 5% level than CPE, DEC and the negative control. DEE has the potential to be antimicrobial exhibiting greatest ZOI, 12.1, 12.2, 12.8 and 16.2 mm against *E. coli,* MSSA, MRSA and VREF, respectively. At the lowest concentration, 5 $\mu g\ \mu L^{-1}$, DEE showed inhibitory effects which are not significantly different from the higher concentrations 10-30 $\mu g\ \mu L^{-1}$ (Table 2). Results of this study support previous findings that *Z. zanthoxyloides*' have antibacterial activities. These studies reported ethanol and methanol extracts' antibacterial activities towards *E. faecalis, B. cereus, B. subtilis* and *P. aeroginosa*. In the same studies, antibacterial activities against three strains (different to the ones used in the present study) of *S. aureus* were also reported (Adebiyi *et al*., 2009; Adegbolagun and Olukemi, 2010). On the other hand, *Z. zanthoxyloides*' chemical and chromatographic fractions were reported to have

Table 1: Comparison of extract mean zones of inhibition for each microorganism

	Mean zone of inhibition (mm)			
Extracts	*E. coli*	MSSA	MRSA	VREF
DMSO	5.963[a]	6.000[a]	6.000[a]	6.000[a]
CPE	7.771[a]	7.711[a]	7.895[a]	6.412[a]
DEE	12.102[b]	12.225[b]	12.749[b]	16.214[d]
DEC	5.944[a]	6.053[a]	8.992[a]	6.049[a]
APC-1	21.606[c]	32.000[d]	6.000[a]	8.474[b]
APC-2	28.488[d]	27.604[c]	34.610[c]	11.898[c]

In a column, Means with the same letters are not significantly different at the 0.05 level by Duncan's Multiple Range Test. DMSO: (negative control); CEE: Crude petroleum ether extract; DEE: Defatted ethanol-ether extract; DEC: Defatted ethanol-chloroform extract APC-1 and APC-2 : Antibiotics (positive controls) in which for *E.coli* are streptomycin and kanamycin; MSSA are penicillin and novobiocin; MRSA are penicillin and novobiocin; VREF are penicillin and chloramphenicol

Table 2: ANOVA for zones of Inhibition (Combined analysis across microorganisms)

Sources of variation	DF	SS	MS	Fc
Block	2	23.687	11.844	1.174
Extract (EX)	3	1968.77	656.257	65.042***
Microorganism (MI)	3	37.304	12.435	1.232
Concentration (CO)	4	73.381	18.345	1.818
EX×CO	12	180.136	15.011	1.488
MI×CO	12	44.121	3.677	0.364
MI×EX	9	248.245	27.583	2.734**
MI×EX×CO	36	168.937	4.693	0.465
Error	154	1553.82	10.09	

**, ***Denote significance at the 0.01 and the 0.001 level, respectively

antimicrobial activities towards *E. coli* (ATCC11775) and *S. aureus* (ATCC 6538) (Odebiyi and Sofowora, 1979).

Interestingly, with DEE tested against VREF, the ZOI, 16.214 mm was significantly greater than those of the two positive controls: (1) penicillin and (2) chloramphenicol, ZOIs, 8.474 mm and 11.898 mm respectively (Table 1). Although linezolid is the antibiotic for VREF (Zeana *et al*., 2001), penicillin and chloramphenicol are known to be inhibitory against gram positive bacteria (i.e., *E. faecium*). DEE's significantly higher potency than either pure penicillin or chloramphenicol in inhibiting VREF could be indicative of DEE containing antibiotic compounds. On the other hand, the zones of inhibition of DEE against *E. coli* (12.102 mm), MSSA (12.225 mm) and MRSA (12.479 mm) were statistically less than the corresponding antibiotics (positive controls) known to be inhibitory against the three test microorganisms (Table 1). The positive controls for *E. coli* were streptomycin, ZOI 21.606 mm and kanamycin, ZOI 28.488 mm. MSSA's positive controls were penicillin, ZOI 32.000 mm and novobiocin, ZOI 27.604 mm. The positive control for MRSA was novobiocin, ZOI 34.61 mm. The ZOI, being significantly higher in the positive controls for *E. coli*, MSSA and MRSA were expected due to the fact that the antibiotics (positive controls) were in pure form; as such could also be of higher concentration than the specific inhibitory compound or compounds present in DEE. Higher antibacterial activities of commercial antibiotics compared to plant extracts have also been reported in other studies (Rojas *et al*., 2006; Doughari and Okafor, 2008; Agbafor *et al*., 2011).

A combined analysis across microorganisms was performed in order to compare microorganisms and their interactions with extracts and concentration levels. From the factors examined in this study namely, extracts, microorganisms and extract concentrations, ANOVA results indicated a significant difference among extracts at the 0.1% level and a significant interaction between extracts and microorganisms at the 1% level. However, no significant difference was observed among the different microorganisms and among the various levels concentrations of extracts used in this study (Table 2). Likewise, no significant interactions were observed between microorganisms and concentrations; microorganisms and extracts and among microorganisms, extracts and concentrations.

Inhibitory activity of defatted ethanol-ether extract (DEE) against vancomycin-resistant *E. faecium*: Figure 2 shows that VREF (16.2 mm zone of inhibition) was significantly most susceptible to DEE compared to MRSA, MSSA and *E. coli* (12.8, 12.2 and 12.1 mm zone of inhibitions). As to why DEE is most inhibitory to VREF compared to MSSA, MRSA and *E. coli* cannot be explained definitively by this study. However, two interesting hypotheses can be forwarded: First, VREF does not have multiple drug resistance (MDR) or efflux pumps to expel the active antimicrobial components of DEE across VREF's membrane. Second, VREF's MDRs for antimicrobials are less efficient. It was reported that both the disruption and the application inhibition of an MDR inhibitor strongly potentiated the antimicrobial action of the plant compound berberine against *S. aureus* (Tegos *et al*., 2002; Hsieh *et al*., 1998).

Results also showed that DEE is broad spectrum which is indicated by its activity against both gram-positive and gram-negative microorganisms. DEE's activity is similar in the gram- positive MSSA, MRSA and the gram-negative *E. coli,* although significantly more active against the gram-positive VREF (Fig. 2). To date, the majority of plant antimicrobials reported were more inhibiting against gram positive compared to gram-negative bacteria (Tegos *et al*., 2002; Butkhup and Samappito, 2011) due to gram-negative bacteria's effective permeability barrier (Tegos *et al*., 2002). Thus there is a need for sources of antimicrobials against gram negative bacteria and DEE will be a promising source of antibiotic against gram-negative bacteria (Butler and Cooper, 2011).

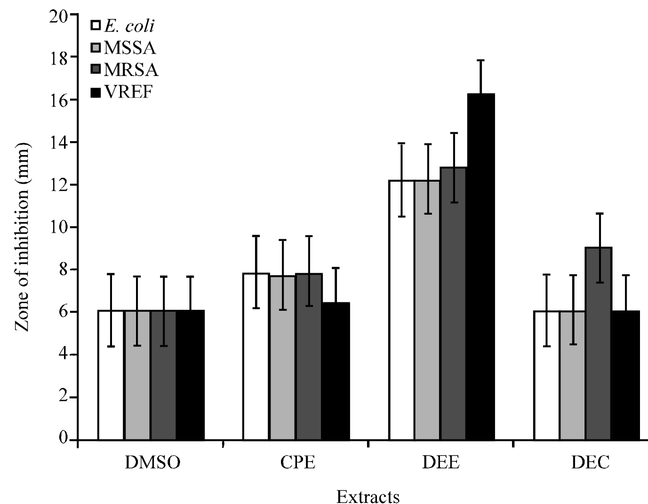

Fig. 2: Bar graphs of the different extract-microorganism combinations with their 95% confidence intervals

Antimicrobial activity of defatted ethanol-ether extract: The antimicrobial property of plant extracts may be related to the presence of some phytochemicals. In DEE extract, thin layer chromatography showed the presence of at least five components. These would more likely be components which are semi-polar owing to its solubility to ethanol and then to ether. These components may include the following: (1) simple phenols and phenolic acids whose mode of action is via enzyme inhibition by the oxidized compounds, (2) quinones which complex with nucleophilic amino acids thereby inactivating proteins, (3) flavones, flavonoids and flavonols have the ability to complex with proteins and bacterial cell walls, (4) tannins inactivate microbial adhesions, enzymes and cell envelope transport proteins and (5) alkaloids activity could be attributed to their ability to intercalate DNA (Cowan, 1999). Phytochemical screening done on *Z. zanthoxyloides* root bark methanolic extracts by Adegbolagun and Olukemi (2010) reported the presence of cardiac glycosides, alkaloids, saponins, tannins and flavonids. On the other hand, Odebiyi and Sofowora (1979) reported the antimicrobial compounds of *Z. zanthoxyloides* to consist of alkaloids namely 6-canthinone, chelerythrine, berberine and phenolic acids (Odebiyi and Sofowora, 1979). In the present study, preliminary and confirmatory tests (Mayer's, Wagner's, Draggendorff's) on the defatted root bark extracted with ethanol prior to ether extraction have shown positive for alkaloids indicated by the observed heavy precipitation or flocculation. Fagaronine, a benzophenanthridine alkaloid with antiplasmodial activity (Kassim *et al.*, 2005; Gansane *et al.*, 2010) and zanthoxylol, a phenolic compound with insecticidal activity (Udo, 2011) could also be candidate compounds responsible for the antimicrobial activities observed in this study.

CONCLUSIONS

Based on the results of this study, Defatted Ethanol Ether extract (DEE) of *Z. zanthoxyloides* should be analyzed further because of its potential as a source of broad spectrum antimicrobial compounds. More importantly, this extract can be a source of compounds which can be used for treating infectious diseases caused by vancomycin-resistant *E. faecium* and methicillin-resistant *S. aureus*. DEE was shown to be inhibitory at the lowest concentration (5 µg μL^{-1}) tested in this study. Thus, future studies will include the determination of the minimum inhibitory concentration against the different microorganisms in this study to further establish the potential of *Z. zanthoxyloides* DEE as an antimicrobial. Furthermore, a bioassay-oriented fractionation DEE to isolate the pure compounds responsible for the observed antimicrobial activities should be conducted. Likewise, it will also be imperative to test if the compounds present in DEE act optimally individually or in a synergistic manner.

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